REMARKS

Claims 2, 5-8, 16-18, 35, 37, and 39-41 will be pending after the entry of the amendment with this response. Claims 1, 11, 12, 19, 22, 25, 26, 33, 34, 36, and 38 have been canceled without prejudice in the interest of advancing prosecution.

Objection to specification

The examiner maintained the previously stated objection to specification for referencing patent applications, specifically for the disclosure at page 25, line 25. A patent has now issued from the referenced application, and the specification has been amended accordingly.

Objections to Claims

The examiner maintained the objections to claims 8, 11, 16-17, 19, 22, and 26 for not being limited to the elected species. Claims 11, 19, 22, and 26, have been canceled, rendering rejections as to those claims moot. With regard to claims 8, and 16-17, Applicants maintain the request to hold in abeyance until allowable subject matter is identified.

35 USC §112, first paragraph

Claims 1, 2, 5-8, 11, 12, 16-19, 22, 25, 26, 33-38 and 39 are rejected as allegedly not enabled by the disclosure of the specification for the full scope of the claims. In response, claims 1, 11, 12, 19, 22, 25, 26, 33, 34, 36, and 38 have been canceled without prejudice to Applicants' right to pursue those subject matter in another application, rendering rejections as to those claims moot. Claims 2, 5-8, 35, and 37 have been amended. Claim 2 was amended to incorporate elements of certain canceled dependent claims, and to more precisely describe the subject matter of the invention. Specifically, claim 2 has been amended to recite particular compounds rather than classes of compounds that reduce the inhibition of a morphogen. The claim has also been amended to recite dendritic growth rather than cell growth in general. Claims 5-8, 35 and 37 have been amended so that they properly depend from pending independent claims.

Applicants submit that the amended claims are supported by the specification, and respectfully request that the rejection on this ground be withdrawn.

35 USC §112, second paragraph

Claims 1, 2, 5, 8, 11, 12, 19, 22, 25, 26, 33-41 are rejected as allegedly being indefinite. In response, claim 41 has been amended to recite "Osteogenic Protein 1" as the full appellation of "OP-1". With regard to the Examiner's assertion that cultured neurons do not express morphogens endogenously, Applicants respectfully traverse and bring the Examiner's attention to the attached references. For example, Wang et al., *Br. J. Oral Maxillofac. Surg.* 2006 July 27 (e-pub ahead of print)(Exhibit A), describes in the abstract how the expression of BMP-2 increased in the neurons of facial nuclei after injury. Setoguchi et al., *Brain Res.* 2001 Dec 7; 921 (1-2): 219-225(Exhibit B) describes in the abstract how BMP7 is expressed in glial cells and motor neurons. Chen et al., *Eur. J. Neurosci.* 2005 Oct; 22(8): 1895-906(Exhibit C) describes in the abstract that BMP4 was expressed in neurons, glial cells, and certain astrocytes. Further, Beck et al., *BMC Neuroscience* 2001, 2:12(Exhibit D) describes cultured sympathetic neurons express BMP-5. Therefore, based on the ample evidence that endogenous morphogens can be found in neurons, Applicants respectfully request that the rejection on this ground be withdrawn.

In view of the above amendment, applicant believes the pending application is in condition for allowance.

Applicant believes no additional fee is due with this response. However, if an additional fee is due, please charge our Deposit Account No. 18-1945, under Order No. JJJ-P01-569 from which the undersigned is authorized to draw.

Dated: August 23, 2006

Respectfully submitted,

By_____

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A

1: Br J Oral Maxillofac Surg. 2006 Jul 27; [Epub ahead of print]

The role of bone morphogenetic protein-2 in vivo in regeneration of peripheral nerves.

Wang YL, Wang DZ, Nie X, Lei DL, Liu YP, Zhang YJ, Suwa F, Tamada Y, Fang YR, Jin Y.

Department of Oral Maxillofacial Surgery, Hua Xi Stomatology Hospital, Sichuan University, Cheng Du 610041, P.R. China; Department of Oral Histology and Pathology, College of Stomatology, Fourth Military Medical University, Xi'an, Shaanxi 710032, P.R. China; Department of Oral Maxillofacial Surgery, Qin Du Stomatology Hospital, Fourth Military Medical University, Xi'an 710032, P.R. China.

We investigated the effects of bone morphogenetic protein-2 (BMP-2) and some other BMPs on regeneration of peripheral motor nerves in vivo. The facial nerves of 24 New Zealand rabbits were crushed to examine a series of retrograde changes in the facial nuclei and axons, in what has been called the "axon reaction". The facial nerves of the experimental group were treated with epineurial coaptation and BMP-2 after the injury. Nerves not treated with BMP-2 were regarded as controls. The expression of BMP-2 was investigated by in situ hybridisation in the neurons of facial nuclei. The electrophysiology, image analysis and transmission electron microscopy were used to evaluate the level of the recovery of facial nerves. The results showed that the axons in the experimental group were thicker and denser than those in the control group four weeks later. The expression of BMP-2 in the neurons of facial nuclei increased after injury. The electron microscopic observations showed that the axons' degeneration in the experimental group was less than that in the control group. Despite the morphological difference between the two groups, there was no apparent difference between them in nerve conduction velocity. These findings suggest that BMP-2 might be involved in the regeneration of facial nerves, and might function as a potential neurotrophic factor.

PMID: 16876296 [PubMed - as supplied by publisher]

Related Links

[The character of glial line-cell derived neurotrophic factor mRNA expression in a facial nerve-striking model] [Hua Xi Kou Qiang Yi Xue Za Zhi. 2002]

Bone morphogenetic protein-7 enhances dendritic growth and receptivity to innervation in cultured hippocampal neurons. [Eur J Neurosci. 2000]

Application of bone morphogenetic proteins in the treatment of clinical oral and maxillofacial osseous defects. [J Bone Joint Surg Am. 2001]

Peripheral nerve explants grafted into the vitreous body of the eye promote the regeneration of retinal ganglion cell axons severed in the optic nerve. [J Neurocytol. 1996]

[Ciliary neurotrophic factor in the regeneration of facial nerve] [Zhonghua Er Bi Yan Hou Ke Za Zhi. 1999]

See all Related Articles...

B

1: Brain Res. 2001 Dec 7;921(1-2):219-25.

Traumatic injury-induced BMP7 expression in the adult rat spinal cord.

Setoguchi T, Yone K, Matsuoka E, Takenouchi H, Nakashima K, Sakou T, Komiya S, Izumo S.

Department of Orthopaedic Surgery, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan. seto@rd5.so-net.ne.jp

It has been reported that bone morphogenetic proteins (BMPs) are involved in the generation of the central nervous system during development. However, the roles of BMPs in mature spinal cord have not been clarified. We examined the expression of BMP7 mRNA before and after traumatic injury of the adult rat spinal cord. BMP7 mRNA was already detectable at a relatively low level in uninjured spinal cord, but was dramatically increased after injury. Semiquantitative RT-PCR study further confirmed upregulation of BMP7 mRNA in injured spinal cord. In situ hybridization indicated that expression of BMP7 mRNA was present only in glial cells in uninjured spinal cord. After injury, the number of BMP7-expressing glial cells was increased, BMP7 expression also became apparent in motor neurons. It has been suggested that BMPs promote survival of subventricular zone cells in adult rats. Thus, our results suggest that increase in the expression of BMP7 promotes survival of neurons and glial cells after acute traumatic injury. In contrast, there is increasing evidence that BMPs inhibit neurogenesis and alternatively promote gliogenesis of neural progenitors, which are also present in adult spinal cord, suggesting that injury-upregulated BMP7 may regulate differentiation of glial cells from neural progenitors and may induce gliosis after central nervous system injury.

PMID: 11720729 [PubMed - indexed for MEDLINE]

1: Eur J Neurosci. 2005 Oct;22(8):1895-906.

Differential expression of cell fate determinants in neurons and glial cells of adult mouse spinal cord after compression injury.

Chen J, **Leong SY**, **Schachner M**.

Zentrum fur Molekulare Neurobiologie, Universitat Hamburg, Martinistrasse 52, 20246 Hamburg, Germany.

Cellular responses after spinal cord injury include activation of astrocytes, degeneration of neurons and oligodendrocytes, and reactions of the ependymal layer and meningeal cells. Because it has been suggested that tissue repair partially recapitulates morphogenesis, we have investigated the expression of several developmentally prominent molecules after spinal cord injury of adult mice where neurogenesis does not occur after injury. Cell fate determinants Numb, Notch-1, Shh and BMPs are abundantly expressed during development but mostly decline in the adult. In the present study, we investigated whether these genes are triggered by spinal cord injury as a sign of attempted recapitulation of development. Expression of Numb, Notch, Shh, BMP2/4 and Msx1/2 was analysed in the adult mouse spinal cord after compression injury by in situ hybridization up to 1 month after injury. The mRNA expression levels of Notch-1, Numb, Shh, BMP4 and Msx2 increased in the grey matter and/or white matter and in the ependyma rostral and caudal to the lesion site after injury. However, BMP2 and Msx1 were not up-regulated. Combining immunohistochemistry of cell type-specific markers with in situ hybridization we found that all the up-regulated genes were expressed in neurons. Moreover, Numb, BMP4 and Msx2 were also expressed by GFAP-positive astrocytes, while Shh was expressed by MBP-positive oligodendrocytes. In conclusion, the cell fate determinants Notch-1, Numb, Shh, BMP4 and Msx2 are expressed in neurons and/or glial cells after injury in a time-dependent manner, suggesting that these genes reflect to some extent an endogenous self-repair potential by recapitulating some features of development.

PMID: 16262629 [PubMed - indexed for MEDLINE]

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Research article

Bone morphogenetic protein-5 (BMP-5) promotes dendritic growth in cultured sympathetic neurons

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Abstract

Background: BMP-5 is expressed in the nervous system throughout development and into adulthood. However its effects on neural tissues are not well defined. BMP-5 is a member of the 60A subgroup of BMPs, other members of which have been shown to stimulate dendritic growth in central and peripheral neurons. We therefore examined the possibility that BMP-5 similarly enhances dendritic growth in cultured sympathetic neurons.

Results: Sympathetic neurons cultured in the absence of serum or glial cells do not form dendrites; however, addition of BMP-5 causes these neurons to extend multiple dendritic processes, which is preceded by an increase in phosphorylation of the Smad-1 transcription factor. The dendrite-promoting activity of BMP-5 is significantly inhibited by the BMP antagonists noggin and follistatin and by a BMPR-IA-Fc chimeric protein. RT-PCR and immunocytochemical analyses indicate that BMP-5 mRNA and protein are expressed in the superior cervical ganglia (SCG) during times of initial growth and rapid expansion of the dendritic arbor.

Conclusions: These data suggest a role for BMP-5 in regulating dendritic growth in sympathetic neurons. The signaling pathway that mediates the dendrite-promoting activity of BMP-5 may involve binding to BMPR-IA and activation of Smad-1, and relative levels of BMP antagonists such as noggin and follistatin may modulate BMP-5 signaling. Since BMP-5 is expressed at relatively high levels not only in the developing but also the adult nervous system, these findings suggest the possibility that BMP-5 regulates dendritic morphology not only in the developing, but also the adult nervous system.

Background

Bone morphogenetic proteins (BMPs) are secreted signaling molecules of the TGF-β superfamily that have been implicated in the control of a host of critical developmental phenomena in the central and peripheral nervous systems [1–3]. BMP-5, one of the more prominently expressed BMPs in the nervous system, has been detected in multiple regions of the nervous system throughout

development and into adulthood [3–6], yet its biological activities in the nervous system are not well defined. A role for BMP-5 in dorsal forebrain patterning has been proposed based on its expression in the dorsal midline of the developing forebrain and observations that ectopic expression of BMP-5 in the developing neural tube of chicks markedly downregulates ventral markers while maintaining dorsal markers [5,7]. Further support for BMP-5 regulation of early forebrain development has been provided by studies of *Bmp5/Bmp7* double mutants [6]. However, reports that BMP-5 in the mouse brain exhibits peak expression levels in the adult striatum and brainstem and that maximal expression in the hippocampus and cerebellum occurs at E18 through PN1 and again in the mature nervous system [3], suggest additional roles for BMP-5 during later stages of neural development and into adulthood.

BMPs have been divided into subgroups based on structural and evolutionary considerations [8]. Although closely related BMPs have been shown to elicit distinct cellular responses [5,9–13], members within a subgroup often display conservation of not only structure, but also function [4–6,14]. BMP-5 belongs to the 60A subgroup of BMPs, which also includes BMP-6/Vgr-1, BMP-7/OP-1, BMP-8a/OP-2, BMP-8b and Drosophila 60A [3,8]. Other members of the 60A subgroup have been shown to modulate neuronal morphogenesis through selective effects on dendrites. Thus, BMPs 6, 7, and 60A stimulate dendritic growth in cultured sympathetic neurons derived from either perinatal or adult ganglia in the absence of effects on cell survival or axonal growth [15–17]. BMP-7 has also been shown to enhance dendritic growth in hippocampal, cortical and spinal motor neurons [18–20].

Whether BMP-5 similarly promotes dendritic growth has not been previously addressed. Since dendrites are the primary site of synapse formation, we felt it was important to examine this possibility. Moreover, since dendritic remodeling occurs throughout the life of the animal, such studies could suggest a function for BMP-5 in the adult nervous system. In this report, we demonstrate that like other members of the 60A subgroup, BMP-5 triggers robust dendritic growth in sympathetic neurons *in vitro* coincident with activation of Smad-1. Noggin and follistatin, soluble proteins known to function as physiological antagonists for BMP-7 [21], also inhibit the dendrite-promoting activity of BMP-5. Furthermore, BMP-5 mRNA and protein are detected in intact sympathetic ganglia and neuron/glia cocultures, respectively, consistent with a proposed role for BMP-5 in regulating dendritic growth in sympathetic neurons *in vivo*.

Results

BMP-5 induces dendritic growth in cultured sympathetic neurons

As previously reported [16], sympathetic neurons grown in the absence of serum and glial cells fail to form dendrites (Figure 1A); however when grown in the presence of BMP-7, these neurons extend multiple dendrites (Figure 1B). Similarly, addition of recombinant human BMP-5 to the culture medium elicits dendritic growth in sympathetic neurons as evidenced by the presence of tapered processes that are immunoreactive for MAP2 (Figure 1C). Comparative analyses of concentration-effect relationships for BMP-7 and BMP-5 (Figure 2), indicate EC_{50} values for BMP-5 approximately 10-fold higher than those of BMP-7; however maximally effective concentrations of these two growth factors have comparable effects on sympathetic neurons as assessed by the percentage of neurons with dendritic growth, the number of dendrites per cell, and the total dendritic length per cell. Time course studies of dendritic growth in neurons exposed to maximally effective concentrations of BMP-5 and BMP-7 reveal comparable temporal responses as well (Figure 3). Both BMP-5 and BMP-7 elicit dendrite formation in 90–100% of the neuronal cell population within 24 to 48 hr (Figure 3A). The gradual increase in the number of dendrites per cell throughout a 17-day treatment with BMP-5 parallels that observed in sister cultures exposed to BMP-7 during the same period (Figure 3B). Experiments in which cultured sympathetic neurons were treated simultaneously with maximally effective concentrations of BMP-5 and BMP-7 indicate that the effects of BMP-5 and BMP-7 on dendritic growth are not additive (data not shown).

Treatment of sympathetic neurons with BMP-5 induces phosphorylation of Smad1

In most cell systems that have been examined, the primary signal transduction pathway for BMPs involves phosphorylation of Smad-1 [22], and recent evidence suggests that this is also true for BMP-7 induced dendritic growth in sympathetic neurons [23]. To determine if BMP-5 initiates similar signaling events in sympathetic neurons, cultured sympathetic neurons grown in the absence of glial cells were treated with BMP-5 for 30 or 60 min then analyzed by Western blotting using Abs specific for total Smad-1 or for phosphorylated Smad-1. Phosphorylation of Smad-1 is evident within 30 min after exposure to BMP-5 (Figure 4A) with significantly increased pSmad-1 levels apparent at 60 min. In contrast, treatment with BMP-5 did not alter levels of total Smad-1 (Figure 4B).

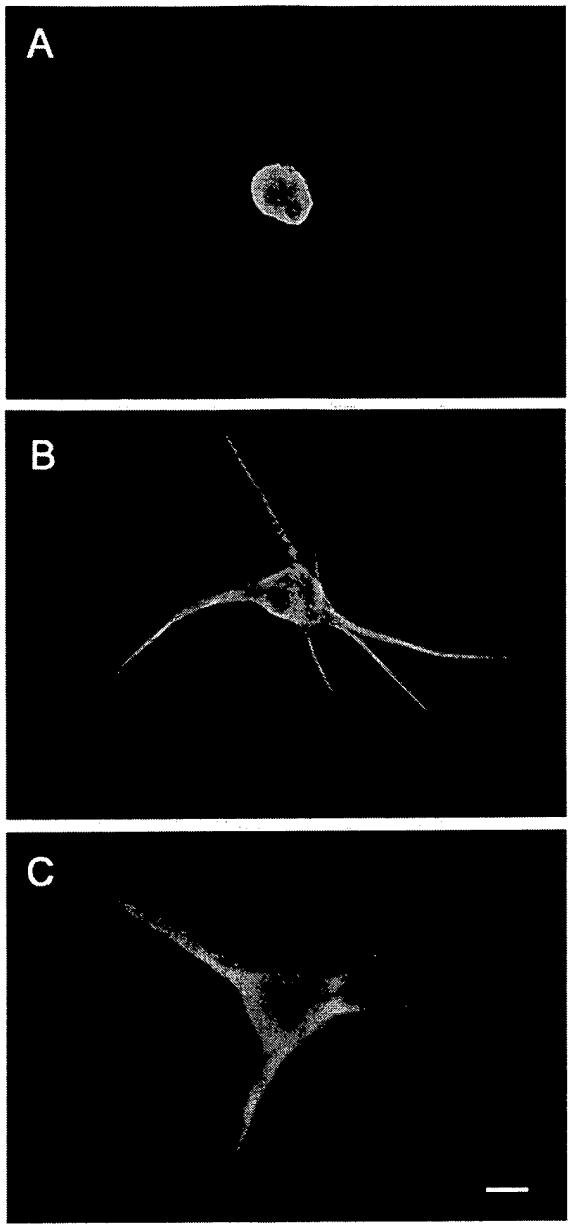

Figure 1
BMP-5 promotes dendritic growth in cultured sympathetic neurons. Non-neuronal cells were eliminated from SCG cultures by treatment with anti-mitotic agent during the 2nd and 3rd days in vitro. Beginning on day 5, sympathetic neurons were treated with either control medium (A), medium supplemented with 50 ng/ml BMP-7 (B), or medium containing 300 ng/ml BMP-5 (C). Six days later, cultures were immunostained with mAb against MAP2, a protein found primarily in dendrites and neuronal somata. Neurons grown under control conditions lack dendrites (A). In contrast, neurons exposed to BMP-7 (B) or BMP-5 (C) typically have several tapered dendrites. Bar, 50 μm.

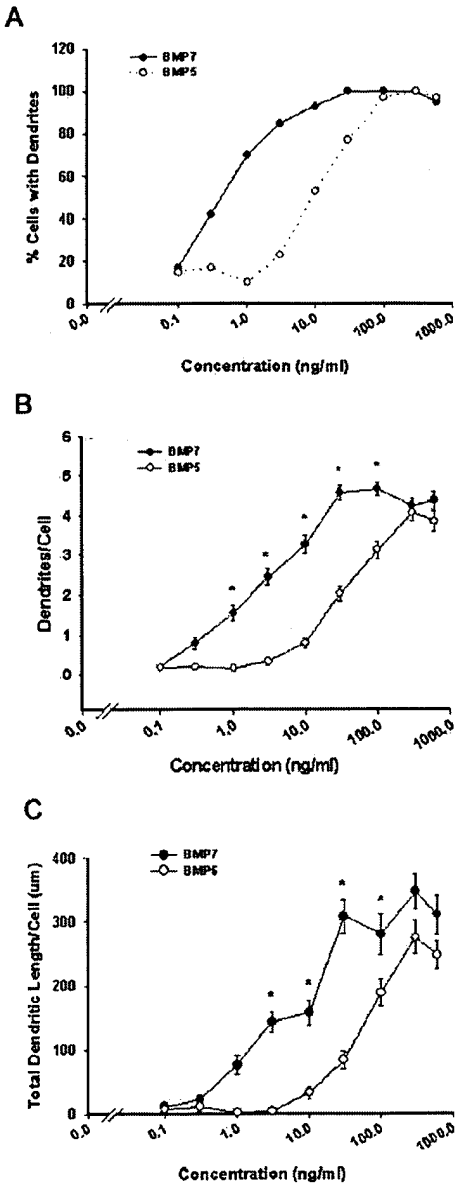

Figure 2
BMP-5 is less potent than BMP-7 but equally efficacious in promoting dendritic growth in cultured sympathetic neurons. Beginning on the fifth day in vitro after elimination of glial cells, SCG neurons were exposed to varying concentrations of BMP-5 or BMP-7. After 6 days of exposure to BMPs, cultures were immunostained with a mAb to the dendritic marker MAP2. Dendritic growth was quantified with respect to the percent of cells with dendrites (A), number of dendrites per neuron (B) and total dendritic length (C). Data in panels B and C are presented as the mean ± S.E.M. (n = 60 per experimental condition). * $p < 0.05$.

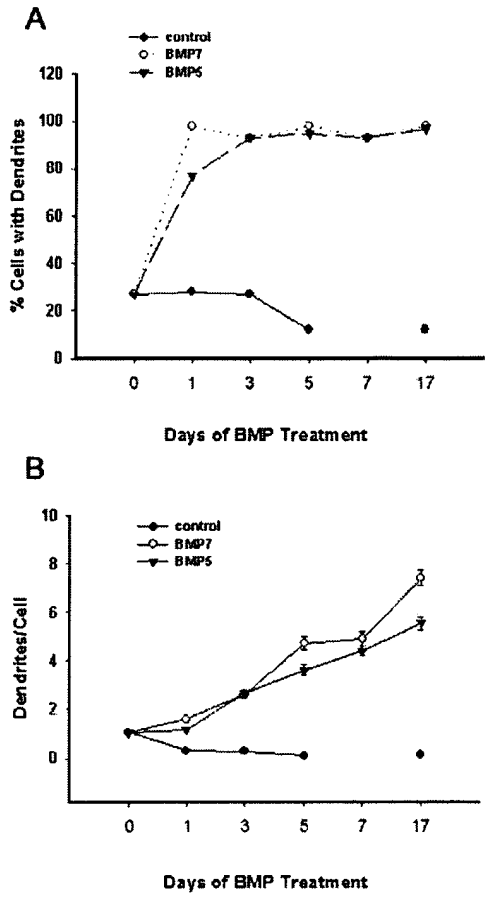

Figure 3
The time course of dendritic growth is comparable between maximally effective concentrations of BMP-5 and BMP-7. Beginning on the fifth day in vitro, SCG neurons grown in the absence of glial cells were treated with control medium or medium supplemented with maximally effective concentrations of BMP-5 (300 ng/ml) or BMP-7 (50 ng/ml). After varying exposure times, cultures were fixed and immunostained with mAb to the dendritic marker MAP2. Dendritic growth was quantified with respect to the percent of cells with dendrites (A) and the number of dendrites per neuron (B). Data in panel B are expressed as the mean ± S.E.M. (n = 60 per experimental condition). There were no significant differences between BMP-7 and BMP-5 at any time point at $p < 0.05$.

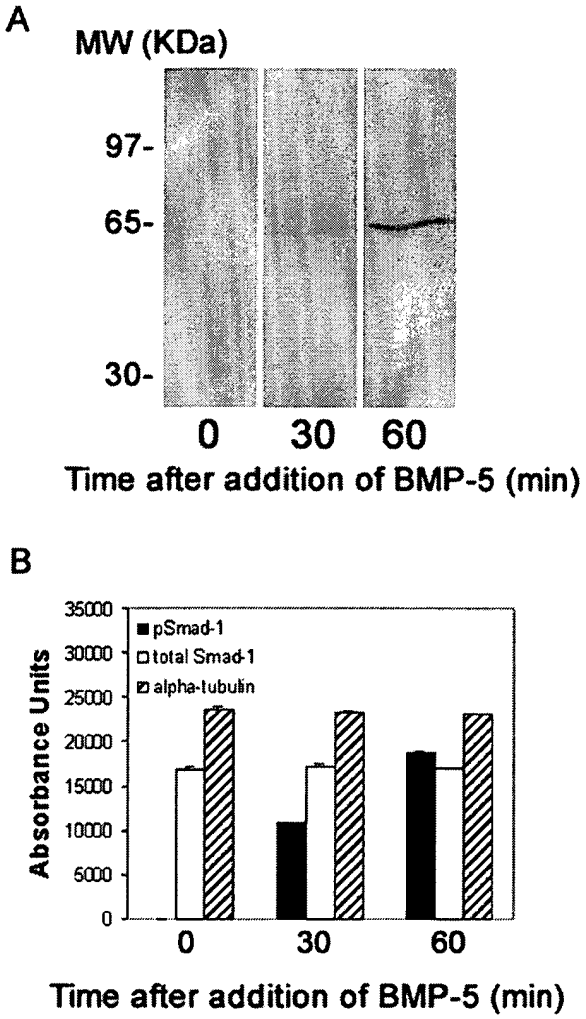

Figure 4
BMP-5 induces phosphorylation of Smad-1 in cultured sympathetic neurons. (A) Blots of cell lysates from SCG cultures consisting solely of neuronal cells were probed using Ab that specifically recognizes the phosphorylated form of Smad-1. Under control conditions (time = 0 min), phosphorylated Smad-1 is not detected. Treatment with BMP-5 (100 ng/ml) causes a time-dependent increase in the band density of phosphorylated Smad-1. (B) Densitometric analyses of Western blots of cell lysates from purified neuronal cell cultures at varying times after exposure to BMP-5. Equal amounts of protein were loaded into all wells and each blot was probed initially for p-Smad, then stripped and successively probed for total Smad and tubulin. Data are presented as the mean ± S.E.M. (n = 2 per experimental condition).

Antagonists of BMP- function inhibit BMP-5-induced dendritic growth

Recent data suggest that in the developing nervous system, signaling by at least BMPs 2, 4 and 7 is determined by the spatiotemporal expression patterns of BMPs and their receptors, and by relative levels of the soluble BMP antagonists such as follistatin and noggin that directly bind BMPs in the extracellular compartment and prevent functional receptor/ligand interaction [1,24–28]. The extent to which these factors may regulate BMP-5 signaling is difficult to determine because of the paucity of data regarding either antagonism of BMP-5 signaling by follistatin and noggin or the interaction between known BMP receptors and BMP-5. To address the former question, we tested the ability of follistatin and noggin to block the dendrite-promoting activity of BMP-5. The latter was evaluated indirectly using a recombinant chimeric protein containing the extracellular domain of the BMP receptor type IA fused to the immunoglobulin Fc domain (BMPR-IA-Fc). The chimeric protein containing BMPR-IA was chosen because it has been previously reported that this is the predominant BMP receptor type expressed in embryonic and postnatal superior cervical ganglia (SCG) [29]. As indicated in Figure 5, follistatin and noggin significantly inhibit the dendrite promoting activity of BMP-5 in a concentration-dependent manner. Maximally effective concentrations of noggin (1000 ng/ml) and follistatin (1000 ng/ml) reduce BMP-5-induced dendritic growth by 77% and 72% respectively. The BMPR-IA-Fc chimera also significantly inhibited BMP-5-induced dendritic growth. Increasing the concentration of any of these antagonists to levels greater than those expressed in Figure 5 did not cause greater inhibition of BMP-5-induced dendritic growth (data not shown).

Expression of BMP-5 mRNA and protein in SCG cells

If BMP-5 is a physiological regulator of dendritic morphogenesis in sympathetic neurons, then its spatiotemporal patterns of expression should be coincident with dendritic growth in this neuronal cell type. Potential sources of BMP-5 include neuronal and glial cells within the ganglia. To examine this possibility, antibody (Ab) specific for BMP-5 was used to immunostain cocultures of SCG neurons and glial cell that had been cultured for 2 weeks to allow for significant expansion of the glial cell population. The specificity of the BMP-5 Ab used in these experiments is indicated by demonstrations that it recognizes purified recombinant BMP-5, but not purified recombinant BMP-6 or BMP-7 in Western blots (Figure 6). Moreover, the reaction with BMP-5 protein is significantly blocked by preincubation of the BMP-5 Ab with blocking peptide (Figure 6). In SCG cultures immunostained with this BMP-5 Ab, immunoreactivity is present in most if not all ganglionic glial and neuronal cells (Fig-

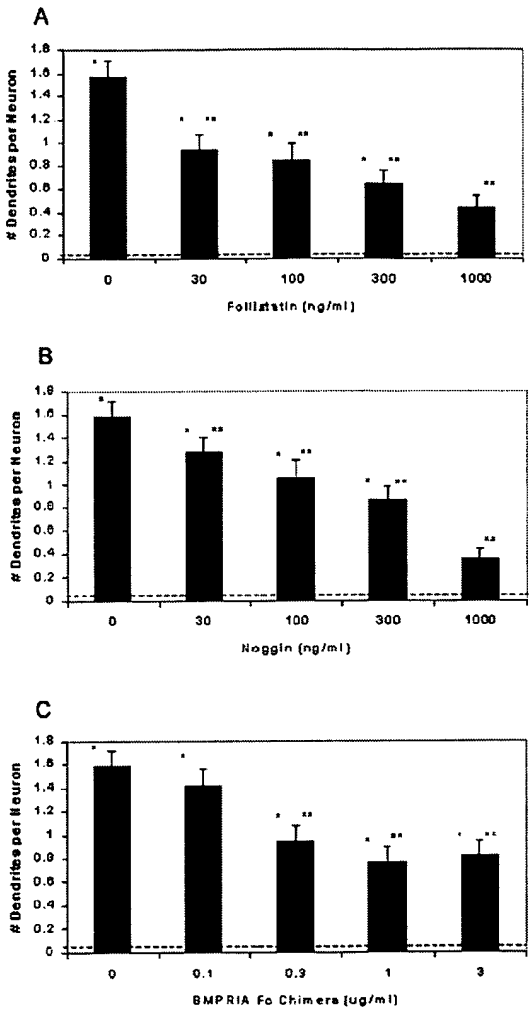

Figure 5
Soluble BMP antagonists inhibit BMP-5-induced dendritic growth. Beginning on the fifth day in vitro, SCG neurons were exposed to BMP-5 (100 ng/ml) in the absence or presence of varying concentrations of follistatin (A), noggin (B) or the BMP-RIA-Fc chimera (C). On the tenth day in vitro, cultures were immunostained with mAb to nonphosphorylated forms of the M and H neurofilaments to visualize dendrites. Dendritic growth was quantified with respect to the number of dendrites per neuron expressed as the mean ± S.E.M. (n = 50 per experimental condition). * Indicates a significant difference from negative control values (- BMP-5/- antagonist) indicated by the dashed line in each bar graph at $p < 0.01$ and ** from positive control values (+ BMP-5/- antagonist) at $p < 0.01$.

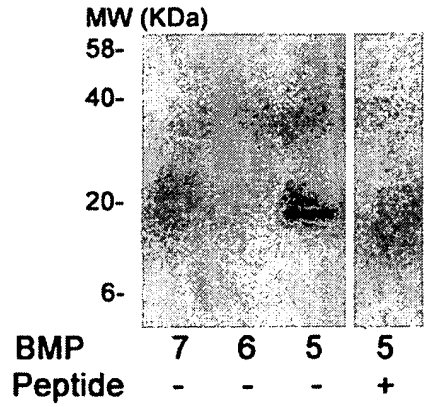

Figure 6
The specificity of the BMP-5 Ab used in these studies as assessed by Western blot analysis. BMP-5 Ab (0.5 μg/ml) reacts with purified recombinant BMP-5 (50 ng), but not with equal amounts of purified recombinant BMP-6 or -7. Preincubation of BMP-5 Ab with specific blocking peptide (2.5 μg/ml) significantly reduces binding to BMP-5 protein. Data represented in this figure were obtained from the same immunoblot.

ure 7A and 7B). In glial cells, fluorescence is present throughout the cell but excluded from the nucleus; in neurons, immunoreactivity is evident in both the soma and processes. Preincubation of the BMP-5 Ab with specific blocking peptide (Figure 7C and 7D) reduced BMP-5 immunoreactivity to levels comparable to those observed in control cultures reacted with the secondary Ab only (Figure 7E and 7F).

BMP-5 expression in vivo was assessed by RT-PCR analyses of SCG harvested from rats of different ages corresponding to various stages of dendritic growth in sympathetic neurons. As indicated in Figure 8, BMP-5 mRNA is present in SCG at embryonic day 20 (E20), which corresponds to the period of initial extension of primary dendrites, and at postnatal days 3 and 7 (PN3 and PN7), which corresponds to the period of maximal dendritic growth. In contrast, BMP-5 mRNA was not detected in adult SCG.

Discussion

BMP-5 is widely expressed in the nervous system throughout development and into adulthood [3–6], yet the only function described for this growth factor thus far is dorsal patterning of the developing forebrain [5–7]. Our data suggest that BMP-5 may also regulate later stages of neural development, specifically dendritic morphogenesis. The most direct support for this hypothesis is the finding that addition of BMP-5 to sympathetic neurons in culture causes these cells to extend multiple dendritic processes. These data are consistent with conclusions from previous studies indicating that dendrite-promoting activity is restricted to BMPs from the 60A or dpp (BMPs 2 and 4) subgroups and is not observed with BMPs from other subgroups such as BMP-3, BMP-13 or dorsalin, or with other members of the TGF-β superfamily such as activin, TGF-β1, -β2 or -β3 [16,17]. Functional redundancy between BMPs of the 60A subgroup has been previously reported with respect to other developmental endpoints [6], of which some, such as upregulation of cell adhesion molecules [30], may be directly relevant to effects on dendritic growth. BMP-6 and BMP-7 as well as dpp subgroup members, BMP-2 and BMP-4, have been shown to influence other aspects of sympathetic neuron development, such as differentiation of adrenergic sympathetic neurons from neural crest [13,31–38] and neuropeptide phenotype [39]. It will be of interest to determine if BMP-5 also exhibits functional redundancy with respect to these effects.

Pharmacological studies of BMP-5 indicate that relative to BMP-7, BMP-5 is less potent but equally efficacious in promoting dendritic growth in cultured sympathetic neurons. These data, together with observations that maximally effective concentrations of BMP-5 and BMP-7 are not additive, suggest that the two ligands share aspects of a common signaling pathway. It has been shown that phosphorylation of Smad-1 precedes dendritic extension in cultured sympathetic neurons exposed to BMP-7; moreover, expression of a dominant negative construct of Smad-1 in cultured sympathetic neurons significantly inhibits BMP-7-induced dendritic growth in these neurons [23]. These data suggest that activation of Smad-1 is a necessary component of the signal transduction pathway by which BMP-7 induces dendritic growth. In this report we demonstrate that BMP-5 similarly induces Smad-1 phosphorylation in sympathetic neurons as detected by Western blot analyses using antibodies specific for the phosphorylated form of Smad-1. These data suggest conserved mechanisms of signaling within the 60A subgroup. The molecular mechanism(s) of BMP-induced dendritic growth downstream of Smad-1 activation are not well characterized. Previous studies have demonstrated that transcriptional and translational events are required for dendritic growth in response to BMPs [15], but the gene expression profile responsible for BMP-induced dendritic growth has yet to be determined. Thus, it is not clear if BMP-5 or -7 induces dendritic growth directly, or if some other factor made by the cells in response to BMPs is responsible for initiating dendritic growth.

BMPs activate Smad-1 by binding to type I and type II serine-threonine kinase receptors [40,41]. Specific re-

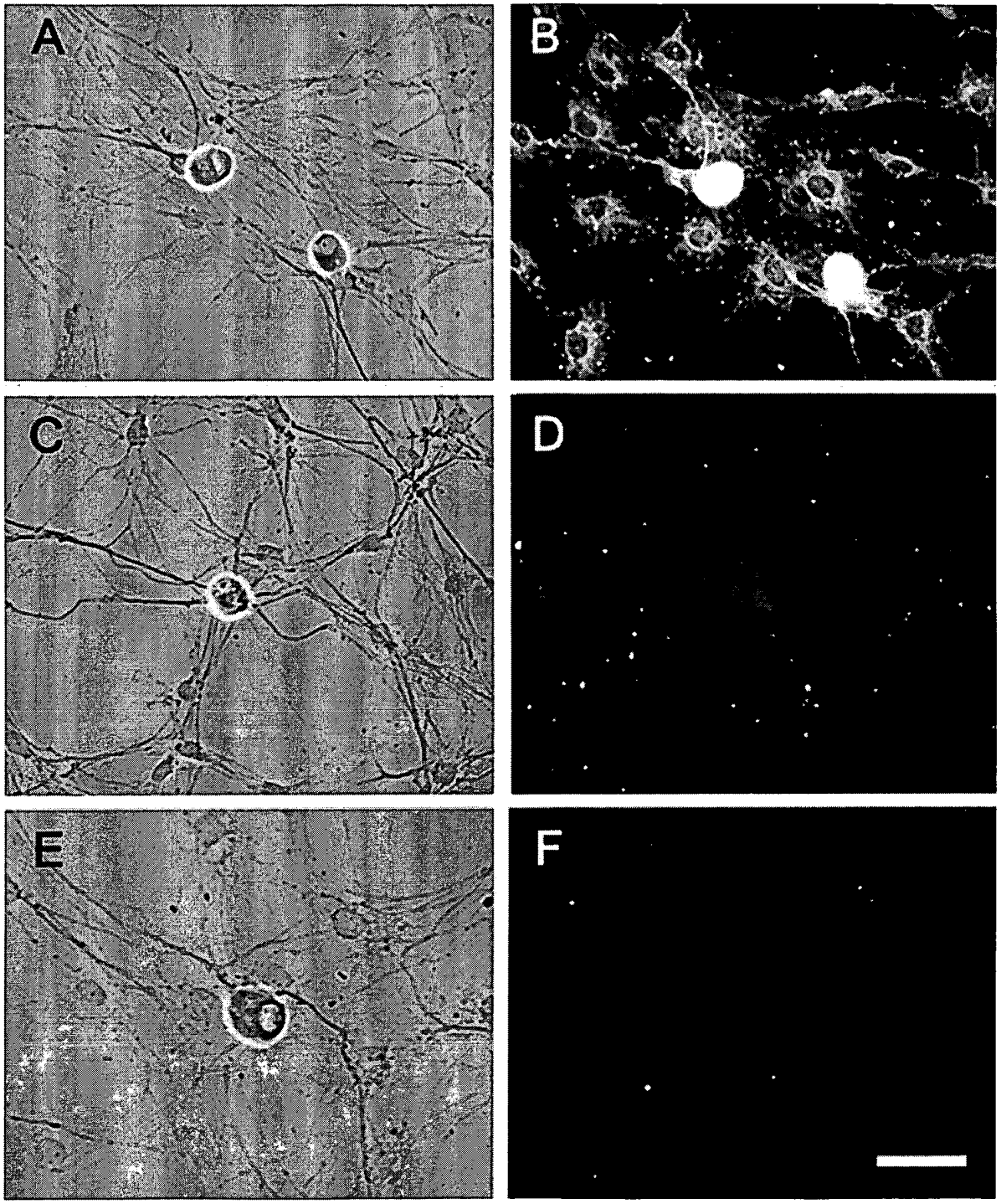

Figure 7
BMP-5 immunoreactivity is detected in co-cultures of SCG neurons and glia. Phase contrast (A,C,E) and fluorescence (B,D,F) micrographs of SCG cultures immunostained for BMP-5 after 10 days in vitro. Both glial cells and neurons express significant BMP-5 immuno-reactivity, and in neurons, the processes as well as the soma are brightly stained (A,B). Preincubation of BMP-5 Ab with blocking peptide prior to reaction with SCG cultures (C,D) reduces the intensity of immunostaining to levels comparable to the background fluorescence observed in SCG cultures reacted only with secondary Ab (E,F).

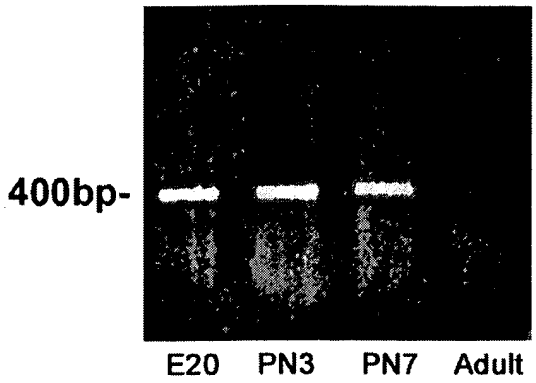

Figure 8
BMP-5 mRNA is expressed in SCG at times corresponding to maximal dendritic growth in SCG neurons. BMP-5 mRNA was detected by RT-PCR in total RNA extracted from rats SCG at E20, PN3 and PN7, which correspond to times of initial dendrite extension (E20) and maximal expansion of the dendritic arbor (PN3 and PN7). In contrast, PCR product was not detected in equal amounts of total RNA isolated from adult SCG.

ceptor subunits shown to bind BMPs include BMP receptor type IA (BMPR-IA), BMPR-IB, BMR-II, activin receptor type I (ActR-I), and ActR-II [42–44]. BMP ligands can bind to either type I or type II receptor subunits independently, but both receptor types are required for high-affinity binding and signaling [40]. The finding that the soluble BMPR-IA-Fc chimera significantly inhibits BMP-5 induced dendritic growth suggests that BMP-5 may be activating the Smad-1 signaling pathway via interactions with BMPR-IA. Although the physiological relevance of this finding has yet to be confirmed by ligand binding studies using endogenous neuronal BMPR-IA, these data are consistent with reports that BMPR-IA is the predominant BMP receptor type expressed in embryonic and postnatal superior cervical ganglia (SCG) [29].

It is becoming increasingly evident that BMP signaling is regulated not only by the spatiotemporal expression of BMP ligands and receptors, but also by relative levels of soluble BMP antagonists, which directly bind BMPs and prevent functional receptor/ligand interaction [1,24–28]. The different BMP antagonists bind to BMPs and other TGF-β family members with varying degrees of specificity. For example, follistatin binds both activin and BMP-7 avidly but competes weakly or not at all with the type I receptor for BMP-4 binding [25,45], whereas noggin binds to BMPs -2 and -4 with greater affinity than BMP-7 [46]. Whether BMP-5 function can be antagonized by noggin or follistatin has not been previously reported, but our results suggest that simultaneous addition of either antagonist with BMP-5 significantly inhibits the dendrite-promoting activity of BMP-5 in a concentration-dependent manner. Thus, profiling the BMP binding affinities as well as the expression patterns of these BMP antagonists will be critical to understanding the regulation of BMP-5 signaling in the nervous system.

If BMP-5 is important in regulating dendritic growth in intact ganglia, its expression should correlate with periods of dendritic growth *in vivo*. In sympathetic ganglia, dendritic growth begins prenatally and maximal expansion of the dendritic arbor occurs during postnatal weeks 1 and 2 [47,48]. RT-PCR analyses of intact SCG indicate that cells of the SCG express BMP-5 transcripts from E20 through P7. Preliminary observations indicate that these BMP-5 transcripts are translated into protein in SCG in vivo as assessed by Western blot analyses using BMP-5 Ab (P. Lein, unpublished observations). Earlier studies have demonstrated the expression of BMP-4 transcripts in developing avian sympathetic ganglia [49], suggesting the presence of multiple BMPs in sympathetic ganglia throughout development. These data, in conjunction with observations that mRNA for BMP type IA and type II receptors are expressed in the developing sympathetic ganglia [29], are consistent with a potential role for BMP-5 in regulating the initiation and rapid expansion of the dendritic arbor in sympathetic ganglia of perinatal animals.

The cellular distribution of BMP-5 was determined by immunocytochemistry in sympathetic neurons cocultured with ganglionic glial cells. Both neurons and glial cells express BMP-5 protein. In situ hybridization analyses of BMP-6 and BMP-7 indicate that both cell types also express BMP transcripts (P. Lein, unpublished observations). These findings are consistent with previous reports that dendritic growth can be induced in sympathetic neurons in vitro when cultured at high neuronal cell density [50] or in the presence of ganglionic glial cells [51].

Dendritic growth continues, albeit to a lesser extent, into adulthood, and dendritic remodeling occurs throughout the animal's life. Mature sympathetic neurons cultured from adult animals respond to BMP-7 with enhanced dendritic growth [15], and treatment with BMP-7 enhances recovery in animal models of stroke [52–56]. Although BMP-5 mRNA was not detected in adult SCG, Western blot analyses indicate that BMP-5 protein is present in adult SCG (P. Lein, unpublished observations), presumably derived from nonganglionic sources

such as serum or target tissues. These observations together with reports that BMP-5 is expressed at significant levels in the adult nervous system [3] suggest a potential role for BMP-5 in modulating dendritic morphology not only during development, but also in adult nervous systems.

Conclusions

These data suggest that BMP-5 regulates dendritic growth. Addition of BMP-5 to sympathetic neurons in culture triggers significant dendritic growth that is concentration-dependent. Data from western blot analyses using Ab specific for phosphorylated epitopes of Smad-1 as well as analyses of dendritic growth in cultures exposed to BMP-5 in the presence of a soluble BMPR-IA-Fc chimeric protein are consistent with a signaling pathway that involves binding to the BMPR-IA and activation of Smad-1. BMP-5 signaling may be modulated by noggin and follistatin since these BMP antagonists were observed to inhibit the dendrite-promoting activity of BMP-5. Spatiotemporal patterns of BMP-5 expression at the mRNA level, as assessed by RT-PCR, and the protein level, as determined by immunocytochemistry correspond to periods of initial dendritic growth and rapid expansion of the dendritic arbor. These observations, together with previously published reports from other laboratories indicating significant levels of BMP-5 expression in the developing and adult nervous system [3] suggest a potential role for BMP-5 in modulating dendritic morphology not only during development, but also in adult nervous systems.

Materials and Methods

Materials

Purified human recombinant BMPs (5, 6 and 7) were prepared using previously published methods [57] and provided by Creative Biomolecules (Hopkinton, MA). Affinity-purified polyclonal antibody (Ab) specific for BMP-5, the blocking peptide for the BMP-5 Ab, and the recombinant human BMP-RIA-Fc chimera were purchased from Research Diagnostics (Flanders, NJ). Ab specific for the phosphorylated form of Smad-1 (Ser 463/465) as well as Ab that recognizes both phosphorylated and nonphosphorylated Smad-1 (e.g., total Smad-1) was purchased from Upstate Biological (Lake Placid, NY). *Xenopus* noggin protein [58] was the generous gift of Drs. Josè de Jesús and Richard Harland (UC at Berkeley). Recombinant human follistatin (B4384) was obtained through Dr. A.F. Parlow at the NHPP, NIDDK (Torrance, CA).

Tissue culture

Sympathetic neurons were dissociated from the SCG of perinatal (E21 to PN1) Holtzmann rats (Harlan Sprague-Dawley, Rockford, IL) according to previously described methods [59]. Cells were plated onto glass coverslips (for immunocytochemical and morphological studies) or 35 mm plastic culture dishes (for Western blot analyses) precoated with 100 μg/ml poly-D-lysine (Sigma, St. Louis, MO). Cultures were maintained in serum-free medium supplemented with β-NGF (100 ng/ml), bovine serum albumin (500 μg/ml), insulin (10 μg/ml), and transferrin (20 μg/ml). In most experiments, endogenous non-neuronal cells were eliminated from cultures by adding cytosine-β-D-arabinofuranoside (Sigma, St. Louis, MO) to the culture medium at 1 μM for 48 hr beginning on day 2. In some experiments this antimitotic was not added to cultures but rather endogenous non-neuronal cells were allowed to proliferate. Previous studies have demonstrated that under these culture conditions, the non-neuronal cells are primarily ganglionic glia [51].

Morphological analyses

Dendritic growth was assessed in cultures immunostained with monoclonal antibodies (mAb) previously shown to react selectively with the somatodendritic compartment of cultured sympathetic neurons [16]. These mAb's include SMI-52, which is specific for the cytoskeletal protein MAP2, and SMI-32, which reacts with the non-phosphorylated forms of the M and H neurofilament subunits (Sternberger Immunocytochemicals, Baltimore, MD). Antigens were localized by indirect immunofluorescence as previously described [16]. Dendritic growth was quantified using SPOT image analysis system. Data in the text are presented as the mean ± S.E.M. and statistical significance was determined using ANOVA followed by Tukey's test.

Western blot analyses

Western blot analyses were performed on purified recombinant BMPs to assess the specificity of the BMP-5 Ab, and on cell lysates of cultured sympathetic neurons to assess the effects of BMP-5 on phosphorylation of Smad1 as well as levels of total Smad (nonphosphorylated and phosphorylated). Cell lysates were obtained by rinsing 12-day old neuronal cell cultures with ice-cold phosphate-buffered saline prior to trituration in ice-cold lysis buffer (PBS supplemented with 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF and 300 μg/ml aprotinin). Cell lysates were microfuged at maximum speed for 5 min and the resultant supernatant collected. Protein concentration was determined using the Bradford assay (BioRad, Hercules, CA). Samples containing equivalent amounts of protein were resolved by 12% polyacrylamide SDS PAGE under reducing conditions and then electroblotted onto PVDF membranes. Blots were blocked at room temperature for 1 hour in TBS-T (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween-20) containing 5% dried fat-free milk, then incubated

overnight at 4°C in TBS-T containing 0.5% milk and primary Ab (0.5 μg/ml for BMP-5 Ab; 10 μg/ml for Smad-1 Ab). Blots were washed twice with TBS-T containing 0.5% milk, then incubated at room temperature for 2 hours in TBS-T containing 0.5% milk and 1:5000 dilutions of secondary Ab conjugated to peroxidase (for BMP-5, anti-goat IgG-peroxidase from Chemicon, Temecula CA; for Smad1, anti-rabbit Ig-peroxidase from Amersham, Piscataway, NJ). Subsequently, blots were washed three times as described above, and visualized using an enhanced chemiluminescence detection method (ECL, Amersham). Blots of cell lysates were stripped and reprobed using antibodies specific for α-tubulin (1:10,000, Sigma). To quantify data, films were scanned using an HP ScanJet ADF scanner and HP Precision ScanPro software, and band density determined as arbitrary absorption units using the MacBas software program (version 2.31, Fuji Film).

Immunocytochemistry

BMP-5 Ab was used to localize BMP-5 protein in SCG cultures containing sympathetic neurons and ganglionic glial cells. After 2 weeks in culture, cells were fixed in 4% paraformaldehyde, permeabilized with methanol at -20°C (Sigma, St. Louis, MO), and then reacted with anti-BMP-5 Ab (10 μg/ml). Immunoreactivity was visualized by indirect immunofluorescence as previously described [16]. The specificity of the immunoreaction was determined by preincubating the BMP-5 Ab with its specific blocking peptide (100 μg/ml) prior to reaction with the cultures.

RNA Isolation and Analyses

Total RNA was extracted from freshly harvested superior cervical ganglia (SCG) using Trizol (Life Technology, Carlsbad, CA). RNA samples (5 μg) were reverse transcribed using random primers at annealing temperatures of 65°C (You-Prime-the-First-Strand kit, Amersham, Piscataway, NJ). Resultant cDNA was amplified by PCR for 35 cycles using an annealing temperature of 55°C for 30 sec and denaturing temperature of 95°C for 30 sec; the Mg^{++} concentration in these reactions was 1.5 mM. As a negative control, each sample was run through PCR without prior reverse-transcription. Primers used for amplification of BMP-5 cDNA were designed to unique sequences of rat BMP-5 using the Primer3 program [http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi] . The specific primer sequences were BMP-5 sense, 5'-TTATGCAAAAGGAGGCTTGG-3' and BMP-5 antisense, 5'-TCATGACCATGTCAGCATCA-3'. After synthesis, PCR products were subjected to 1% agarose gel electrophoresis and found to have the expected size of 420 base pairs.

List of abbreviations

Ab, polyclonal antibody

BMP, bone morphogenetic protein

BMPR-IA, BMP receptor type IA

E20 or E21, embryonic day 20 or 21

mAb, monoclonal antibody

PN1, PN3 or PN7, postnatal day 1, 3 or 7

SCG, superior cervical ganglia

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